Embryogenesis and neogenesis of the endocrine pancreas

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CHAPTER 7

Summary, general discussion and future perspective

SUMMARY

The present study was undertaken to study the expression of nestin, c-met and c-kit in the embryogenesis and the development and growth of the adult pancreas. The rationality for doing these studies was that these proteins were included in the development of the pancreas and of other organ structure and some of them had even been proposed to be present on precursor cells. To confirm that these populations were indeed involved in growth mechanisms in the pancreas we decided to do a robust experiment. We infused pure populations of c-met and c-kit carrying cells isolated from the neonatal pancreas into a diabetic rat pancreas and studied the effect on glycemic parameters and the weight gain in the rats (chapter 3). This experiment demonstrated that in these populations of cells, some cells may have curative effects. This, however, does not implicate that these cells are precursor cells, since, as shown in chapters 4, 5, and 6, our populations of cells is heterogeneous. It only shows that the infused populations of c-met and c-kit carrying cells contain some cells which may have a supporting effect on regenerative processes or which may develop into beta-cells.

To further study these cells, we decided to study in more detail the location of these cells in the developing and growing endocrine and exocrine pancreas.

In chapter 4 of this thesis we investigated the localization of nestin, c-met, and c-kit positive cells in the endocrine, *i.e.* in the islets, and in the exocrine, *i.e.* the ductules, tissue, and in blood vessels, in the embryonic, neonatal and physiological growing, *i.e.* pregnant, rat pancreas. During the embryonic stage, both nestin and c-met, but not c-kit were shown to be present on some specific populations of cells. Also during the post-natal stage, nestin, c-met, and c-kit positive cells were present, although their numbers decreased in the adult stage. This illustrates that these proteins are involved in development and differentiation of the immature pancreas to the adult state. Both the location of the proteins and the stage of development suggest that the cells expressing these proteins are recruited for development of the pancreas. Also we found that the number of cells expressing nestin, c-met, and c-kit in the pancreas is influenced by the presence of a metabolic challenge such as during pregnancy, in which the endocrine pancreas grows. This suggests that these

cells are also recruited into the growing pancreas and that they play a role in the growth process.

In chapter 5 we further characterized the population of cells that express nestin, c-met, and c-kit in the neonatal pancreas, *i.e.* the population of cells infused in chapter 3 and show that these are heterogeneous populations of cells. Our results showed that nestin, c-met and c-kit positive cells express proteins specific for pancreatic (precursor) cells (Islet-1, ngn3, Pax4, Pax6, amylase, Pdx-1, insulin, PP/SOM, and glucagon), endothelial cells (CD31) and nerve cells (β3-tubulin).

In chapter 6 we did a separate experiment in order to study whether the cells expressing c-met and c-kit could be forced to express insulin by growth factor manipulation. After 10 days of culture in medium containing beta-cell specific growth factors, differentiated c-met cells appeared to be in a more immature beta-cells stage than differentiated c-kit cells, since the former cells still express Pax6 and Pax4, whereas in differentiated c-kit cells the expression of these transcription factors disappeared. This demonstrates that this heterogeneous population of cells with a specific unique lineage restricted differentiation can be forced to redirect their differentiation and gain a more beta-cell like phenotype. However, since we were dealing with heterogeneous populations of cells, it cannot be excluded that the manipulation, resulted in proliferation of specific populations and apoptosis of others and therefore preferential growth of some population above others. Also clonally expansion of specific populations may be involved. These questions have to be addressed in subsequent studies.

GENERAL DISCUSSION

In this thesis we show that nestin, c-met, and c-kit are proteins that are involved in development of the pancreas. Nestin and c-met are abundantly present on embryonic and foetal cells and in the adult pancreas and in the adult pancreas during pregnancy. Surprisingly, c-kit is observed in all stages except for the foetal stage. As c-kit is the receptor for the so-called stem-cell factor and is mainly associated with expression on bone-marrow derived stem cells [1] it is tempting to speculate that in all stages except for the foetal stage bone-marrow derived stem cells are involved in growth

processes of the pancreas. This, however, might be specific for the rat as in the foetal pancreas c-kit expressing cells are abundantly observed [2-4].

Our study shows that the cells expressing nestin, c-met, and c-kit express the transcription factors Pax4, Pax6, pdx-1, insulin, glucagon and amylase. Our immunocytochemical analysis showed that the cells indeed were cells with characteristics of beta-cells, alpha-cells, and exocrine cells. However, the proteins were not only found on pancreatic cells, but they were also found on CD31 and β3-tubulin expressing cells, *i.e.* markers that are specific for endothelial cells and nerve cells, respectively.

Next to our finding that nestin, c-met and c-kit positive cells represent different functional cell-populations, *i.e.* they show different gene expression profiles, these cells appear to be present in variable numbers in time. We observed, as expected with cells involved in development and growth, that during development of the pancreas more precursor cells are present than in the adult or adult growing pancreas.

Preliminary results in our *in vivo* model have shown that c-kit positive cells have the capacity to lower blood glucose levels. Due to technical limitations it was not possible to study *ex vivo* nestin positive cells and their capacity to differentiate toward beta-cells. However, previous studies using nestin positive cells have shown that these cells have the capacity to differentiate into insulin producing cells, most likely beta-cells [5]. Based on these and our own findings, showing that nestin positive cells are present in the islets of the developing and growing pancreas and that they express pdx-1 and ngn3 which are crucial for beta-cell differentiation, one could hypothesize that also nestin positive cells have a functional role in the development or regenerating pancreas.

Based on our observations it can be questioned whether nestin, c-met and c-kit positive cells have the same appearance during the whole process of development and regeneration. It is obvious and of course expected that these cells change their protein expression over time. For instance, a nestin positive cell may at a later time point in differentiation loose its nestin expression and gain either c-met or c-kit expression, or both. This may result in a different cell, but in reality it is still the same, originally nestin positive, cell that changed its phenotypical appearance.

Most likely this change of protein characteristics will be reflected in changes in function.

From our studies, including chapter 6 it should not be interpreted that we conclude that our isolated and purified population of nestin, c-met, and c-kit expressing cells are precursor cells. It is a heterogeneous population of cells with cells in all stages of development and is probably even expressed in cells that undergo transdifferentiation or normal mitosis. It has, however, been shown by Suzuki *et al.* [2] that c-met expressing cells contain minor population of cells with unlimited proliferative capacity and some characteristics of stem cells. These cells with more multipotent than pluripotent characteristics may also be present in the purified populations of c-met and c-kit expressing cells, but we did not yet do any studies in this direction. The scientific proof for the presence of precursor cells in our populations of cells should come from clonal expansion experiments, which will be performed in the near future. On the basis of our own results we can only conclude that all three studied proteins are expressed in a timely manner in specific stages of growth on cells involved in supporting functions and possibly on differentiating cells. The proteins are not exclusively expressed on insulin producing cells, but at least the heterogenous population of c-met expressing cells can be forced to produce insulin.

FUTURE PERSPECTIVES

To address the issues described in the general discussion, tools can be developed for better selection/isolation, differentiation, and application of pancreatic cells. In our experimental set-up we have made use of Fluorescence Activated Cell Sorting (FACS) based sorting of cells using antibody-ligand staining. In our experiments we were only able to isolated c-met or c-kit positive cells, since the proteins c-met or c-kit are expressed in the membrane of the cells. Although we have studied nestin positive cells, we were not able to isolate these cells from the pancreas and perform culture studies on them, since nestin is an intracellular protein which does not allow FACS based cell sorting without compromising their viability. In order to circumvent this technical problem one could try to find others methods to isolate nestin positive cells from the neonatal pancreas. This could be done by developing

rats expressing Enhanced Green Fluorescent Protein (EGFP) under the control of the nestin promoter, ideally without disturbing endogenous nestin production. This would result in animals that specifically express EGFP in nestin positive cells. These EGFP expressing nestin positive cells could then be easily isolated and sorted prior to further research.

In our experiments we have studied heterogeneous populations of pancreatic cells expressing nestin, c-met, and c-kit. In order to conclude that precursor cells are present it is mandatory to do clonal expansion studies. These experiments are also required to conclude whether any of the proteins studied in the present thesis have any implications for isolation of precursor cells, for example, for islet-cells banking for transplantation purposes.

In addition to the optimalisation of selection or differentiation of pancreatic precursor cells, a number of other possibilities are open when it comes to the application of these cells in *in vivo* systems. In this thesis we have performed pilot experiments regarding the infusion of pancreatic precursor cells into the damaged pancreas. Possibly, experiments can be performed in which the infused cells remain for a longer period of time in the animals, before taken the animals out of the experiment. Another suggestion would be to perform infusion experiments in which the effect of the number of infused cells is examined.

In a nutshell, on the basis of the present findings we have shown that (sub-) population of the cells expressing nestin, c-met, and c-kit are heterogeneous populations and may be involved in embryogenesis and neogenesis of the pancreas. Our findings clearly show that cells in the population of nestin, c-kit and c-met positive cells precursor cells may be present, since we found the expression of endocrine specific genes like ngn3, islet-1, pax4 and pdx-1.This suggests that (some) nestin, c-met and c-kit expressing cells may play a role in the formation of endocrine cells, in particular insulin producing beta-cells. Although, c-met and c-kit expressing cells seem to have a pancreatic precursor cell geno- or phenotype, our data showed that *in vitro* and *in vivo* both cell populations behaved somewhat different than we expected. *In vitro* c-met expressing cells can be manipulated to produce insulin and *in vivo* c-kit expressing cells have the capacity to lower blood glucose levels. From these data, it seems that c-kit expressing cells may

be better candidates for achieving normoglycaemia than c-met expressing cells. The reason for the difference in behaviour needs to be further investigated. In this thesis we have investigated the localisation, phenotypic and genotypic appearance, differentiation capacity of nestin, c-met and c-kit expressing cells. Like others, we have also shown that these cells can be used as potential candidates for the formation of beta-cells. However, before these cells can be used in human stem cell therapy we need to have a clear and thorough picture about their characteristics and their behaviour *in vitro* and *in vivo*. The questions that were raised in the beginning of this thesis: *What are the requirements to manipulate a stem cell to become a fully functional insulin producing beta-cell? Which biological factors are involved in the end-stage differentiation of a precursor towards a fully glucose responsive beta-cell? How can senescence and genetic disorders like unlimited proliferation in stem cells be avoided?* still stand ground. Some of our data even let to more questions such as: *What is the optimal transplantation site for nestin, c-met and c-kit positive cells? Should these cells be transplanted into the pancreas or can they also be placed in the ontology related organs, like the liver?* These questions still need to be investigated and understood in detail before we can use nestin, c-met and c-kit expressing cells in clinical setting as therapeutic tools for the treatment, or even cure, of diabetes mellitus or other pancreas related diseases.

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